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RESEARCH ARTICLE

Bevacizumab as a monoclonal antibody inhibits mitochondrial complex II in isolated rat heart mitochondria: ameliorative effect of ellagic acid

Elham Mohammad Khanlou[a,b], Saman Atashbar[a,b], Farzad Kahrizi[c], Nima Shokouhi Sabet[a,b] and Ahmad Salimi[a]

[a]Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran; [b]; [c]Department of Toxicology and Pharmacology, Faculty of Pharmacy, Damghan Islamic Azad University, Damghan, Iran

ABSTRACT

Drug-induced cardiotoxicity usually manifests as heart failure or left ventricular systolic dysfunction. Left ventricular dysfunction is a rarely reported side effect of bevacizumab (BEV) with an incidence of 1.2%, and this occurs irrespective of the route of administration. In this study, we focused on an analysis of BEV effects on mitochondrial complexes activities and protective effect of ellagic acid (EA) against BEV-induced mitochondria toxicity. Rat heart mitochondria were isolated using differential centrifugation form wistar rats. Using biochemical and flowcytometry assays we evaluated mitochondrial complexes activity, succinate dehydrogenases (SDH), mitochondrial swelling, reactive oxygen species (ROS) formation and mitochondrial membrane potential (MMP) in isolated mitochondria. We observed only decreased activity of complexes II after exposure with BEV (50 and 100 μg/ml). The inhibition of complex II is paralleled by the decreased MMP, mitochondrial swelling, and ROS formation. Also, we showed that EA (10–100 μM) as an antioxidant and natural agent significantly decreases mitochondrial toxicity induced by BEV. Together, for the first time, this preliminary study has demonstrated a significant decrease in activity of complexes II after exposure with BEV and proved the protective effects of EA in alleviating BEV-mediated mitochondria toxicity.

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KEYWORDS
Bevacizumab; cardiotoxicity; mitochondria; ellagic acid; antioxidant; Complex II

Introduction

As cancer treatments continue to progress and improve, the adverse cardiovascular side effects events related to cancer treatment have also attracted more attention (Curigliano *et al.* 2016). Antiangiogenic drugs such as bevacizumab (BEV) that have been approved for the treatment of several cancers inhibit hypoxia-inducible factor 1-related signaling and vascular endothelial growth factor (VEGF) (Kerbel 2006). Several adverse cardiovascular effects like left ventricular dysfunction and subsequent heart failure have been reported in the previous studies (Schmidinger *et al.* 2007). Antiangiogenic drugs develop myocardial dysfunction probably on the basis of the heart's dependence on adequate angiogenesis and mitochondria-related signaling pathways (Chu *et al.* 2007). BEV as a recombinant humanized monoclonal antibody shows approximately 2% of development of cardiomyopathy and heart failure in BEV-treated patients (Bordun *et al.* 2015). Large molecules BEV as monoclonal antibody can be internalized through the nonselective process such as macropinocytosis and selective process related to Fc receptor (FcRn) (Lim and Gleeson 2011). Macropinocytosis process is originated from actin-containing plasma membrane extensions, resulting in large endocytic vacuoles called macropinosomes that ultimately become early endosomes (Commisso *et al.* 2013). A recent study on glioblastoma (GBM) cells showed that BEV is internalized rapidly through macropinocytosis into cells and is detected in the lysosomes (Müller-Greven *et al.* 2017). Also, the same internalization and accumulation of BEV were shown in retinal endothelial cells accompanied by interaction with the cytoskeleton (Deissler *et al.* 2016). Their studies suggest that BEV can internalize into cell from different process. Therefore, chances of exposure of mitochondria to BEV in cardiomyocytes are possible.

Although cardiovascular safety concerns of many widely used drugs like BEV have been identified today, the cellular mechanisms of adverse cardiac effects are poorly understood. As well as, the predictive value of now existing toxicity screening methods is very poor, especially in individuals with cardiovascular abnormalities (Madonna *et al.* 2015). Notwithstanding the great attempts to disclose cardiotoxicity in the preclinical phase and clinical trials of development of drugs, cardiotoxicity continues to lead safety concerns. This is clearly owing to the lack of enough knowledge of the mechanisms of cardiotoxicity (Kang 2001). New evidence proposes that cardiotoxicity of drugs usually induce mitochondrial dysfunction (Varga *et al.* 2015). There are adequate reasons to evaluate the role of drugs in inducing mitochondrial impairments and the leading potential toxic effects or mitochondrial dysfunction. Knowledge of these mechanistic studies on mitochondrial may help the clinicians design safer therapeutic regimens in the patients (Pereira *et al.* 2009). In addition, a published work indicated that the predictive

CONTACT Ahmad Salimi salimikd@yahoo.com, a.salimi@pharmacy.arums.ac.ir Department of Toxicology and Pharmacology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran

performance of the mitochondrial assays for cardiotoxicity is 33% (Rana *et al.* 2019).

Numerous studies showed the beneficial effects of natural products against cardiotoxicity. Regardless of sources of antioxidants natural products are known to prevent the progression of cardiac tissue damage (Yu *et al.* 2018). Ellagic acid (EA), a type of natural compound, is widely distributed in plants and has been demonstrated to possess a strong ability to scavenge free radicals. EA has shown anti-apoptosis and anti-inflammatory activities in many systems. Many studies have reported that EA has strong antioxidant activities (Salimi *et al.* 2015, Chen *et al.* 2018). To our knowledge, there are no reports to date about the effect of BEV on mitochondrial complexes and protective effect of EA on BEV-induced mitochondria dysfunction in isolated mitochondria as promising system for perdition of cardiotoxicity. Thus, this study aims to search the effect of BEV on mitochondrial complexes and protective effect of EA on BEV-induced mitochondria toxicity.

Material and methods

Animals

Male wistar rats (8–9 weeks old) weighting 200–300 g were purchased from the Pasteur Institute of Iran (Tehran, Iran). Animals were housed under standard conditions (temperature 22–21 °C, humidity 50–10%, 12 h light–dark cycle and free access to food and water). This study was approved by the Ethics Committee at the Ardabil University of Medical Sciences with ethics code IR.ARUMS.REC.1397.275, and performed strictly in accordance with institutional and international guide for animal care.

Chemicals

Rhodamine123, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), *N*-(2-hydroxyethyl) pi-perazine-*N*′-(2-ethanesulfonic acid) (HEPES), trypan blue, ethylene glycol-bis(β-aminoethyl ether (EGTA), bovine serum albumin (BSA), coomassie brilliant blue, 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA), sucrose, D-mannitol, dimethyl sulfoxide (DMSO), 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monopotassium phosphate, 3-morpholinopropane-1-sulfonic acid (MOPS), sodium succinate, rotenone, magnesium chloride, and potassium chloride were purchased from Sigma (St. Louis, MO) (Cambridge, UK).

Preparation of mitochondria

Rats were decapitated and the heart was surgically extracted. The isolated heart was chopped, cleared from blood vessels, and destroyed with a glass homogenizer in a 10-fold volume of the medium containing 225 mM D-mannitol, 75 mM sucrose, and 0.2 mM EDTA, pH 7.4 in ice-cold bath. The homogenate was centrifuged at 1000×*g* for 10 min, and the pellet was removed. The mitochondria contained in the supernatant were sedimented at 10 000× *g* for 10 min at 4 °C (Odinokova *et al.* 2018). The protein content in mitochondria was determined using the Bradford assay was determined (Bradford 1976). Protein concentration in the suspension was 1 mg/ml.

Spectrophotometric measurement of enzyme activity

The enzymatic activities of oxidative phosphorylation (OXPHOS) complexes I, II, III, IV, I + III, II + III, and citrate synthase were measured in isolated rat heart intact mitochondria according to presented situation in Table 1 (Spinazzi *et al.* 2012). For all assays, enzymatic activity was measured in the presence of 50 and 100 μg/ml BEV for 30 min. Experiments were performed at least three times for each treatment. Significant differences between control and treated samples were determined using one-way ANOVA test (Spinazzi *et al.* 2012).

Measurement of NADH/succinate dehydrogenase activity

The activity of NADH dehydrogenase (complex I) and/or succinate dehydrogenase (complex II), was assayed by using MTT reduction (Hosseini *et al.* 2013). Briefly, after incubation of mitochondrial suspensions with BEV (50 and 100 μg/ml) and EA (10, 50, and 100 μM) at 37 °C for 60 min and then was added 0.4% MTT to the medium, samples were incubated at 37 °C for 30 min. Then, the purple formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm with an ELISA reader (BioTek, Winooski, VT) (Liu *et al.* 1997).

Table 1. Conditions for spectrophotometric assays of respiratory chain enzymes activity in isolated rat heart mitochondria.

	Complex I	Complex II	Complex III	Complex IV	Complex I + III	Complex II + III
λ (nm)	340	600	550	550	550	550
Buffer	KP	KP	KP	KP	KP	KP
pH	7.5	7.5	7.5	7	7.5	7.5
Substrates/ electron acceptors	NADH, 100 μM Ub 1, 60 μM	Succinate, 20 mM DCPIP, 80 μM DUB 50 μM	DubH 2, 100 μM Cyt c, 75 μM	Cyt c H2, 60 μM	NADH, 200 μM Cyt c, 50 μM	Succinate, 10 mM Cyt c, 50 μM
Detergent	–	–	Tween-20 (0.025%, vol/vol)	–	–	–
Specific inhibitor	Rotenone, 10 μM	Malonate, 10 mM	Antimycin A, 10 μg ml^{-1}	KCN, 300 μM	Rotenone, 10 μM	Malonate, 10 mM

λ: selected wavelength for the assay; Cyt c: cytochrome c; Cyt c H2: reduced cytochrome c; DCPIP: 2,6-dichlorophenolindophenol; DUB: decylubiquinone; DubH2: decylubiquinol; DTNB: 5,5′-dithiobis (2-nitrobenzoic acid); KCN: potassium cyanide; KP: potassium phosphate buffer; Ub1: ubiquinone1.

Quantification of mitochondrial ROS formation

After incubation of isolated heart mitochondria with BEV (50 and 100 μg/ml) and EA (10, 50, and 100 μM) in respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4, 5 mM sodium succinate, and 10 μM DCFH-DA). The mitochondrial H_2O_2 production was assayed by flowcytometry (Cyflow Space-Partec, Germany) in the period of 60 min. Mitochondria were read on the FL1 channel of flowcytometry and mean of fluorescence intensities were compared between groups (Eruslanov and Kusmartsev 2010).

Determination of MMP collapse

Rhodamine 123 as cationic fluorescent dye has been used for the determination of MMP collapse. Briefly, mitochondria were suspended in MMP buffer including 220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μM Rotenone, and 10 μM of rhodamine123. Then mitochondria were incubated with BEV (50 and 100 μg/ml) and EA (10, 50, and 100 μM) at 37 °C for 5 min. The fluorescence was measured using flowcytometry (Cyflow Space-Partec, Germany). Mitochondria were read on the FL1 channel of flowcytometry and mean of fluorescence intensities were compared between groups (O'Connor *et al.* 1988).

Determination of mitochondrial swelling

The change in mitochondrial volume, due to the colloidal osmotic effects of solute flux into and out of the mitochondrial matrix was measured by monitoring the absorbance at 540 nm (A_{540}). Briefly, after incubation of mitochondrial suspensions with BEV (50 and 100 μg/ml) and EA (10, 50, and 100 μM) at 37 °C for 5 min in swelling buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, and 1 μM of rotenone), the absorbance was measured at 540 nm at 60 min with an ELISA reader (BioTek, Winooski, VT, USA). A decrease in the absorbance indicates an increase in mitochondrial swelling (Gerencser *et al.* 2008).

Statistical analysis

The results were analyzed using Graph Pad Prism version 5 (Graph Pad Software Inc., La Jolla, CA). Results are presented as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the *post-hoc* Tukey posttest and two-way ANOVA followed by the posttest Bonferonie. Statistical significance was set at $p < 0.05$. Also, the flow cytometric data was obtained with Cyflow Space-Partec and analyzed by FlowJo.

Results

OXPHOS enzymatic activity

OXPHOS enzymatic activity in isolated mitochondria obtained from rat heart was measured. Complex II activity was significantly reduced by BEV at 50 and 100 μg/ml compared with control group (Figure 1(B)). Activities of complexes I, III, and IV were not affected by BEV (Figure 1(A,C,D)). Also complexes I/III and II/III activity were not significantly reduced to control activity by BEV (Figure 1(E,F)). In summary, these results

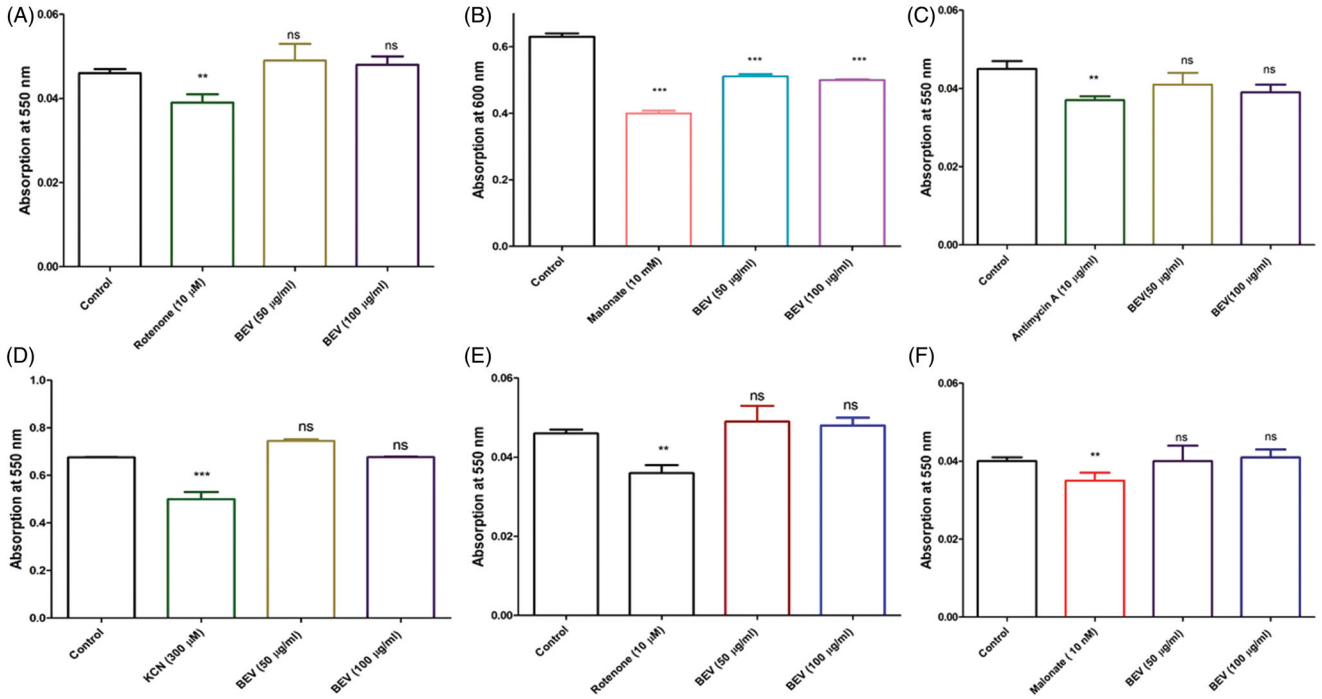

Figure 1. OXPHOS enzyme activities of complex I (A), complex II (B), complex III (C), complex IV (D), complex I + III (E) and complex II + III (F) in isolated rat heart intact mitochondria. Only complex II activity was significantly reduced by bevacizumab at 50 and 100 μg/ml compared with control group but not in other complexes. **and ***indicate $p < 0.01$ and $p < 0.001$ *vs.* control respectively. Three independent experiments were run for each complex.

confirm that complex II is the principal OXPHOS target for BEV in rat heart mitochondria.

Measurement of NADH/succinate dehydrogenase activity

Evaluations of BEV for potential activity on mitochondria obtained from rat heart mitochondria were carried out by studying the inhibitory effects of this drug on NADH/succinate dehydrogenase activity using the MTT assay. BEV (up to 50 μg/ml) inhibited succinate dehydrogenase activity after 1 h of exposure (Figure 2). The presented data at Figure 2 demonstrated that EA (10, 50, and 100 μg/ml) prevent mitochondria toxicity induced by BEV.

ROS formation assay

Reactive oxygen species (ROS) plays a key role in cell survival and death. We evaluated the effect of BEV on ROS formation in isolated rat heart mitochondria using DCFH-DA staining. As shown in Figure 3, treatment with BEV at up to 50 μg/ml at 1 h, significantly ($p < 0.05$) induced ROS generation in isolated rat heart mitochondria. These results suggested that BEV induced ROS generation might antioxidant agents such as EA inhibits the ROS formation induced by this drug. When the isolated mitochondria were simultaneously treated with BEV + EA, the mean fluorescence intensities were significantly decreased compared to treated groups with BEV (Figure 3).

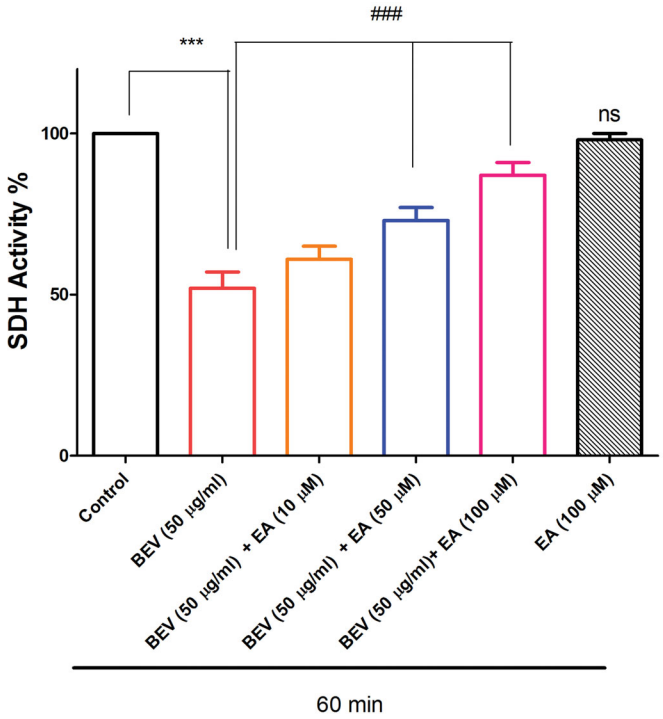

Figure 2. Effect of bevacizumab on succinate dehydrogenase activity and protective effect of EA in isolated rat heart mitochondria. Mitochondrial succinate dehydrogenase activity was measured by MTT assay within 1 h. Presented data showed bevacizumab significantly decreased succinate dehydrogenase activity compared to control. Also, protective effect of EA is tested against mitochondrial toxicity induced bevacizumab. This data showed EA significantly increased succinate dehydrogenase activity compared to treated groups with bevacizumab alone. Values were expressed as mean ± SD of three separate determinations. ***indicates $p < 0.001$ *vs.* control and ###indicates $p < 0.001$ *vs.* treated group with bevacizumab.

Mitochondrial swelling

Induction of mitochondrial swelling in isolated mitochondria was monitored by following 540 nm absorbance (A540) decrease. BEV (up to 50 μg/ml for 1 h) resulted in an extensive mitochondrial swelling in isolated rat heart mitochondria (Figure 4). Also, our result showed that mitochondrial swelling was inhibited after treatment of isolated mitochondria with BEV at toxic doses by EA (10, 50, and 100 μg/ml).

MMP collapse

To search for the identification of mechanisms involved in apoptosis, we examined the effects of BEV on membrane permeability of mitochondria ($\Delta\Psi m$) in isolated rat heart mitochondria. Treatment with of BEV (up to 50 μg/ml for 1 h) induced significant decrease in $\Delta\Psi m$ (Figure 5) in isolated rat heart mitochondria. As shown in Figure 5, collapse of mitochondrial membrane potential (MMP) was inhibited after treatment of mitochondria with BEV by EA (10, 50, and 100 μg/ml).

Discussion

Depending on the reversibility of myocardial damage, there are two types anticancer drug-induced cardiotoxicity. Type I anticancer drugs such as anthracyclines, alkylating agents, and antimicrotubule agents directly cause cell death resulting in irreversible myocyte destruction and congestive heart failure or CHF. On the other hand, type II cardiotoxic anticancer drugs change normal cellular function by affecting the protein synthesis and the mitochondrial system (Jain *et al.* 2017). Type II cardiotoxicity was first described with VEGF inhibitors and tyrosine kinase inhibitors like trastuzumab (Economopoulou *et al.* 2015). Endothelial growth factor inhibitors disrupt the VEGF signaling cascade and induce cardiovascular toxicity. VEGF is a modulator of growth and myocardial function. Therefore, VEGF inhibitors may produce different forms of cardiovascular toxicity, mainly left ventricular dysfunction, thromboembolism (TE), heart failure, and hypertension (Touyz and Herrmann 2018). In mitochondria, VEGF signaling pathway stimulates the angiogenesis by promoting the mitochondrial functions including mitochondrial oxidative respiration, intracellular ATP levels, ROS production decrease, and increase in the enzymes of ROS defense system, including catalase and glutathione peroxidase (GPX1) (Guo *et al.* 2017). Also, VEGF activated mammalian target of rapamycinm (mTOR) signaling pathway to promote the function of mitochondria (Laplante and Sabatini 2012). Therefore, VEGF inhibitors could decrease mitochondrial activity and impair cardiomyocyte function. The myocardium requires appropriate perfusion to function properly and it depends on VEGF and HIF-1 pathways, similar to tumors. Therefore, inhibition of a VEGF may cause microvessel rarefaction and myocardial hibernation, which can be reversed by elimination of the VEGF inhibitor (Ramakrishnan *et al.* 2014). Such dependency shows that the myocardium is very sensitive to anti-angiogenic treatments, like BEV (Ramakrishnan *et al.* 2014). BEV as an VEGF inhibitor, is now widely used for the

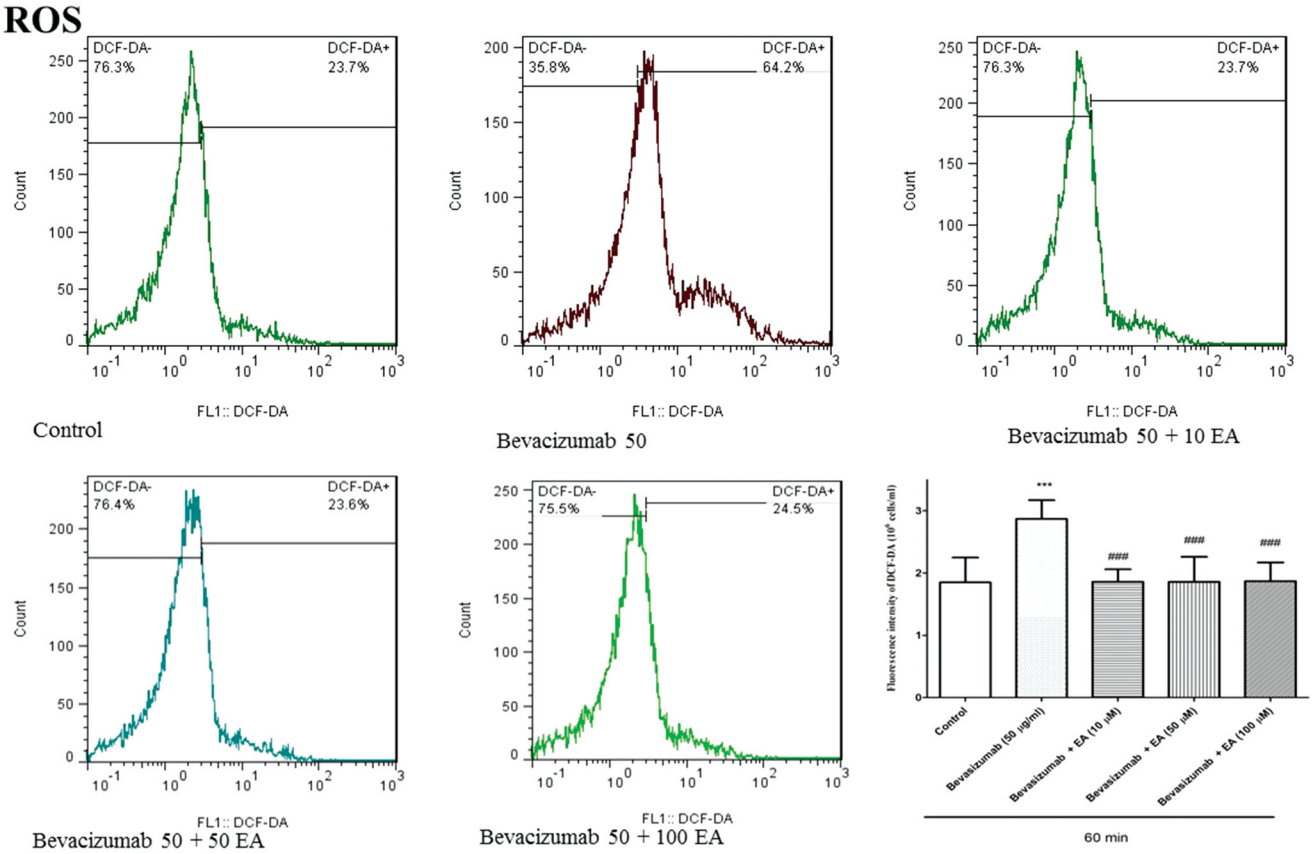

Figure 3. Effect of bevacizumab on ROS formation and protective effect of EA in isolated rat heart mitochondria. Changes in ROS generation were measured in isolated mitochondria after treatment with bevacizumab (50 μg/ml) for 1 h. The fluorescence intensity of DCF was significantly ($p < 0.05$) increased in bevacizumab-treated mitochondria. Also, protective effect of EA is tested against ROS formation induced bevacizumab. This data showed EA significantly decreased ROS formation compared to treated groups with bevacizumab alone. Values were expressed as mean ± SD of three separate determinations. ***indicates $p < 0.001$ *vs.* control and ###indicates $p < 0.001$ *vs.* treated group with bevacizumab.

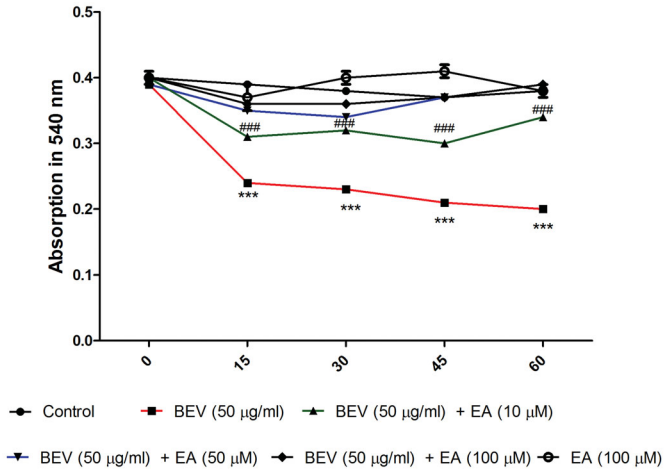

Figure 4. Effect of bevacizumab on mitochondrial swelling and protective effect of EA in isolated rat heart mitochondria. Mitochondrial swelling was monitored by following 540 nm absorbance decrease. Bevacizumab at concentration 50 μg/ml induced mitochondrial swelling in in isolated rat heart mitochondria in a time depending manner. EA significantly inhibited mitochondrial swelling compared to treated groups with bevacizumab alone. Values were expressed as mean ± SD of three separate determinations. ***indicates $p < 0.001$ *vs.* control and ###indicates $p < 0.001$ *vs.* treated group with bevacizumab.

treatment of several cancers. Previous study showed that BEV as a mitochondrial membrane depolarizer had a direct perturbation effect in the mitochondria of primary brain tumors (Nanegrungsunk *et al.* 2016). They showed that BEV -induced mitochondrial depolarization is independent from the ROS pathway since the application of BEV to brain tumor mitochondria did not increase ROS formation (Nanegrungsunk *et al.* 2016). They suggested that probably BEV could directly open the mitochondrial permeability transition pore (mPTP or MPTP), subsequently depolarize the mitochondrial membrane and cause mitochondrial swelling (Nanegrungsunk *et al.* 2016). Moreover, BEV exposure for 5 d was safe at clinical doses in both human retinal pigment epithelial (ARPE-19) and rat neurosensory retinal (R28) cells in culture (Luthra *et al.* 2013). By contrast, BEV exposure at all doses shows a significant dose-dependent decrease in mitochondrial activity in both the proliferating and non-proliferating human microvascular endothelial (HMVEC) *in vitro* (Luthra *et al.* 2013). Obtained results suggest a selective action of BEV on endothelial cells at clinical doses (Luthra *et al.* 2013). Our result for first time proved that BEV has a direct perturbation effect in isolated rat heart mitochondria and causes mitochondrial oxidative stress, permeabilizing the mitochondrial membrane and mitochondrial swelling as the 'point of no return' in the cascade of events leading to cell death.

In many cases, cardiotoxicity problems emerge only in the presence of additional cardiovascular disease conditions or appear months/years following the exposure (Varga *et al.* 2015). Heart isolated mitochondria as a good tool could be more effective in prediction of cardiotoxic agents.

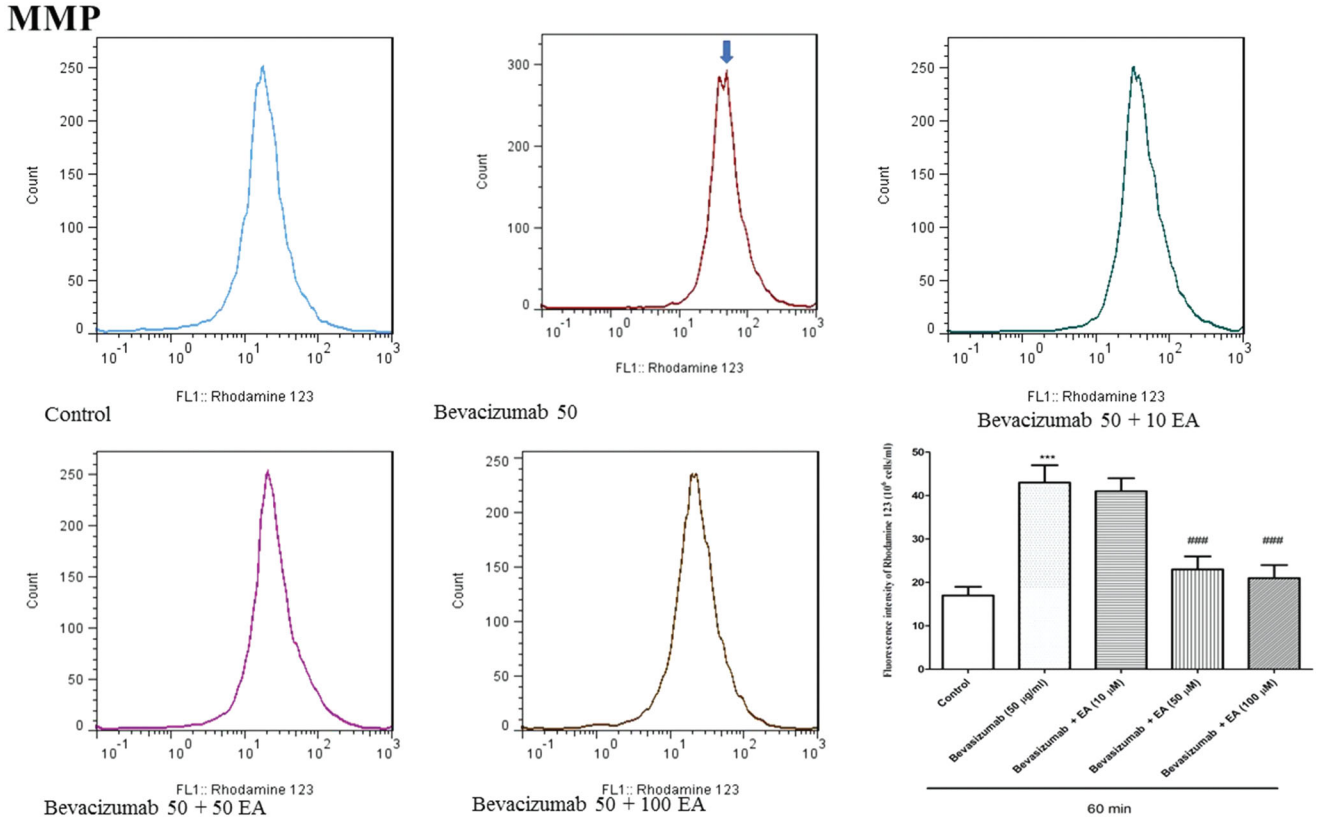

Figure 5. Effect of bevacizumab on ΔΨm and protective effect of EA in isolated rat heart mitochondria. Freshly isolated mitochondria were incubated with 50 µg/ml bevacizumab for 1 h. ΔΨm was measured following rhodamine 123 staining with flow cytometry. The presented data revealed that exposure to bevacizumab caused a significant increase in the fluorescence intensity of rhodamine 123 and it reflects MMP collapse. In addition to this figure shows EA prevented bevacizumab-induced MMP collapse. ***indicates $p < 0.001$ *vs.* control and ###indicates $p < 0.001$ *vs.* treated group with bevacizumab.

In mitochondria, cardiotoxic drugs induce toxicity through different mechanism of actions such as interference with the mitochondrial respiratory chain and/or inhibition of the important mitochondrial enzymes (Varga *et al.* 2015, Gorini *et al.* 2018). The final phase of mitochondrial dysfunction induces an increase in mitochondrial oxidative stress and loss of MMP, eventually lead to cell death (Varga *et al.* 2015, Kuznetsov *et al.* 2011). Our results on isolated mitochondria showed that BEV inhibits mitochondrial complex 2 and probably triggers other mitochondrial toxicity events. The majority of ROS is produced as a by-product of mitochondrial respiration and about 2% of the molecular oxygen is converted into superoxide radicals by mitochondria in normal cells (Zorov *et al.* 2014). Main sites of superoxide generation by mitochondria are complexes I and III (Zhao *et al.* 2019). Besides complexes I and III, succinate dehydrogenase complex (complex II) is also considered to be an important site of excessive superoxide formation, particularly when mitochondrial respiration is suppressed and there is an excess of succinate levels (Scialò *et al.* 2017). In these situations, reverse electron transport occurs to complex I, resulting in extensive uncontrolled ROS formation and the accumulated succinate is quickly reoxidized by complex II, leading to further ROS production (Scialò *et al.* 2017).

Cardiotoxicity induced by drugs and chemicals is closely linked to mitochondrial oxidative stress (Deavall *et al.* 2012). This situation resulted in oxidative stress from the imbalance generation of ROS and antioxidant defense systems. Thereby, oxidative stress contributes to cardiovascular complications and endothelial dysfunction (Incalza *et al.* 2018). Natural products have appeared as a potential source of bioactive compounds which have shown to protect against cardiotoxicity-induced by oxidative stress (Ooi *et al.* 2018, Arbeláez *et al.* 2018). There are many works showed that EA, as a phenolic compound has cardioprotective effect against chemical and drug-induced cardiotoxicity (Warpe *et al.* 2015, Hemmati *et al.* 2018). In this study, we proved that EA has mitochondrial protective effect against toxicity induced by BEV, which is consistent with previously published data examining antioxidant and cardioprotective effects of EA in several systems. A published study suggests that protective effect of EA may be accomplished particularly throughout the mitochondrial maintenance either directly by its antioxidant property or indirectly through its maintaining of complex II (Keshtzar *et al.* 2016). These findings also suggest a potential role for EA in treating or preventing mitochondria associated disorders. In summary, BEV directly induced mitochondrial dysfunction in rat heart mitochondria due to, its ability to induce ROS production through complex II inhibition which lead to permeabilizing the mitochondrial membrane and mitochondrial swelling. Finally, this study suggests a possible usefulness of EA as a mitochondrial protective agent contributing to a safer use of BEV in patient subjected to chemotherapy.

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Disclosure statement

The authors declare that they have no conflict of interest.

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